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Monoclonal antibody specific for the transverse tubular membrane of skeletal muscle activates the dihydropyridine-sensitive Ca^{2+} channel

(reconstitution/dihydropyridine receptor/function-specific monoclonal antibody/immunoprecipitation)

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ABSTRACT **In skeletal muscle, dihydropyridine receptors and dihydropyridine-sensitive Ca^{2+} channels are preferentially localized in the transverse tubular membranes. Starting with an antigenic membrane fraction enriched in rabbit muscle transverse tubules (T-tubules), several monoclonal antibodies were produced by a fusion of spleen cells from an immunized BALB/c mouse with P3 × 63Ag.8.6.5.3 mouse myeloma cells. Antibodies were screened according to a scheme designed to select IgG immunoglobins that recognized a determinant specifically associated with the T-tubule membrane. Antibodies that fulfilled the screening criteria were used in *in vitro* planar bilayer recording of the activity of the dihydropyridine-sensitive Ca^{2+} channel present in T-tubules. Cells producing one antibody (Ab 21) survived cloning dilution and stably produced a monoclonal antibody (mAb21-4) that increased the rate of single channel opening when interacting with the internal side of the channel protein. mAb21-4 immobilized by covalent crosslinking on beads (Affi-Gel 10) consistently immunoprecipitated polypeptide bands with the following electrophoretic mobility: M_r values of ≥ 175,000; 90,000; 55,000; and 34,000.**

Ca^{2+} channels are ubiquitous to almost all cell types and participate in diverse cell functions such as motility, neurotransmitter and hormone release, muscle contraction, and pacemaker activity (1). In skeletal and cardiac muscle, identification and functional reconstitution of Ca^{2+} channels has been possible with the use of dihydropyridine (DHP) agonists and antagonists, a group of structurally related drugs that binds with high affinity to the channel protein and chemically modulates the opening and closing of an otherwise partially inactive Ca^{2+} channel (2–4). Using DHPs as radioligands, several groups (5–8) have recently purified from skeletal muscle of DHP-receptor–complex that has been assumed to be associated with functional Ca^{2+} channels. The DHP receptor of skeletal muscle described by Curtis and Catteral (5) has subunits of ≈ M_r 140,000; 50,000; and 31,000; a similar composition has been reported by others (6–8). The 140,000 protein is the probable site of DHP binding (but see ref. 9) and can be phosphorylated almost stoichiometrically by cAMP kinase (8, 10).

The answer to the question of which protein components of the DHP receptor in skeletal muscle are essential to form a functional Ca^{2+} channel is unclear. Skeletal muscle has a 50- to 100-fold excess of spare DHP receptors not accountable by the number of functional Ca^{2+} channels per cell (11). Likewise, only a small percentage of the purified DHP receptor appears to mediate drug-sensitive Ca^{2+} fluxes in liposomes (3). Thus, a minor component of the purified complex could account for Ca^{2+} channel activity. This, and the long-standing disparity between ligand-binding affinity and electrophysiological potency of the DHPs (8, 11, 12) has limited the use of these drugs as radioligands for the Ca^{2+} channel protein. In the present paper, we pursued an alternate approach to establish the composition of functional Ca^{2+} channels in skeletal muscle. Exploiting the observation that in skeletal muscle, the slow voltage-dependent Ca^{2+} currents are predominantly concentrated in the transverse tubules (T-tubules) (13–15), we have raised monoclonal antibodies (mAbs) against purified T-tubule membrane constituents. We report the production of one mAb (mAb21-4) that recognizes a surface membrane epitope located only at the triad (formed by anatomic coupling of the SR/T tubule membrane), presumably specific for the T-tubule. When screened for functional effects on DHP-sensitive Ca^{2+} channels of T-tubules (2), mAb21-4 markedly increased activity by specifically increasing the rate of single channel opening. mAb21-4 immunoprecipitated several polypeptides with electrophoretic mobility similar, in part, to those from the purified DHP receptors reported by several groups (4–8).

MATERIALS AND METHODS

Isolation of Membranes. The antigenic fraction utilized in the immunization and screening experiments was prepared from a triad-enriched microsomal fraction as previously described (16–18). To obtain T-tubule membranes, triads were disrupted with 0.4 M KCl. The resultant sarcoplasmic reticulum (SR) and T-tubule vesicles were separated according to their differing buoyant density by isopycnic centrifugation (17, 19). The T-tubule membranes were recovered from the 22–27% sucrose region of the gradient with a purity of ≈80%, and the SR membranes were recovered from the 31–36% region of the gradient with a purity of ≈95% (17). [*5-methyl*-^{3}H]PN200-110 (New England Nuclear) binding measurements (16) indicated 60 pmol of high-affinity DHP binding sites per mg of protein. A somewhat less pure surface membrane-enriched fraction (50 pmol of [^{3}H]PN200-110 binding sites per mg of protein) was utilized as a source of protein for the immunoprecipitation studies. This fraction was recovered from the 20–27% region of a sucrose gradient that contained membranes sedimenting at 2600–35,000 × *g* (18).

Production of Antibodies and Screening Assays. Sendai virus-free BALB/c male mice (The Jackson Laboratory)

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Abbreviations: DHP, dihydropyridine; T-tubules, transverse tubules; SR, sarcoplasmic reticulum; mAb, monoclonal antibody; P_i/NaCl, phosphate-buffered saline.

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were immunized with a T-tubule-enriched membrane as described (16).

Hybridomas were screened for the production of IgG isotype by a solid-phase ELISA. Polyvinyl chloride 96-well microplates were sensitized with poly(L-lysine) (10 μg/ml) followed by goat anti-mouse Fc fragment antibody (Jackson ImmunoResearch, Avondale, PA) used at a dilution of 1/1000 in phosphate-buffered saline (P_i/NaCl), pH 7.4. After being washed the plates were incubated with supernatant fluid from the hybrid colonies and extensively washed. Alkaline phosphatase-linked goat anti-mouse Fc antibody (used at 1/1000 dilution in P_i/NaCl) was subsequently overlaid to permit specific binding to IgG isotypes. After being washed with P_i/NaCl, wells were filled with 2,2'-azino-*dl*(3-ethylbenzthiazoline sulfonate) (Cappel Laboratories, Cochranville, PA) solution, and IgG-producing colonies were identified by the enzymatic color reaction. IgG-producing colonies were further screened for those producing antibodies that bound specifically with T-tubule-enriched membrane fractions. All IgG-positive supernates were tested in a second ELISA screen against several fractions of the two different membrane types, respectively enriched in membranes originating in either T-tubules or SR. Immulon plates (Flow Laboratories) were coated first with poly(L-lysine) and incubated overnight with membrane from the respective fraction [1–5 μg/ml in carbonate buffer, pH 9.6 (20)]. Supernates from IgG-producing hybrid colonies were exposed overnight at 4°C to the immobilized individual membrane preparations. A peroxidase-linked goat anti-mouse IgG (Cappel, used at 1/1000 in 0.14 M P_i/NaCl at pH 7.4) was allowed to bind with the primary antibody from the supernatant solutions. Antibodies specific for the T-tubule-enriched membranes were identified and selected after treating the peroxidase with the substrate *p*-nitrophenyl phosphate (Sigma). The IgG-containing supernates were also selected for analysis by immunocytochemistry as described below. Colonies producing the desired antibody were cloned by limiting dilutions.

Immunocytochemical Analysis. Supernates containing antibodies of the IgG isotype were examined against reactivity with the triads as previously described (16) using an avidin–biotin-complex immunoperoxidase stain with a Vectastain staining kit (Vector Laboratories, Burlingame, CA) precisely following the manufacturer's directions. The supernatant fluids containing antibodies to be screened were exposed overnight at 4°C to fixed muscle sections, or for 1 hr to fresh muscle sections. 3-Amino-9-ethylcarbazole was used as a peroxidase substrate (21). Staining was examined by light microscopy. mAbs were isotyped as to immunoglobulin heavy and light chains using a Boehringer Mannheim enzyme-linked immunoassay kit.

Purification of mAb. mAbs were purified on staphylococcal Protein A, covalently linked to Sepharose CL-4B beads (Pharmacia). The mAb was eluted with 0.1 M sodium citrate/citric acid at pH 5.0 (22) and detected at 280 nm. The fractions containing the antibody were collected in 1–2 ml of 0.2 M Tris buffer, pH 7.5, and pooled. The antibody was prepared for physiological studies (see below) by dialyzing it extensively against 0.85% NaCl. Protein concentration was done using bovine serum albumin as a standard (23).

Immunoaffinity Purification. Membrane fractions containing vesicles from T-tubules were solubilized with a buffer (24) of 1.0% (wt/vol) digitonin (Fisher)/185 mM KCl/25 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (Hepes)-NaOH/1 mM EGTA, pH 7.4 containing the following protease inhibitors—0.5 μg of antipain per μl, 0.5 μg of chymostatin per ml, 0.5 μg of pepstatin per ml, 100 units of pertinin per ml, 2 μgm of leupeptin per ml (Sigma) at 4°C for 2–3 hr. The extract was clarified by centrifugation for 1 hr at 100,000 × *g*. The supernate containing the solubilized material was passed overnight over an affinity column. A 0.5-ml bed volume immunoaffinity column was prepared with activated Affi-Gel 10 beads (Bio-Rad) on which mAb21-4 was cross-linked following the manufacturer's specifications. After the column was extensively washed with solubilizing buffer, the antigen was step-eluted with KCl (that increased in ionic strength) in 25 mM Hepes-NaOH, pH 7.4/1 mM EGTA/0.3% digitonin containing protease inhibitors. The eluate between 1 M and 2 M KCl was resolved after disulfide bond reduction with 5% 2-mercaptoethanol by $NaDodSO_4$/PAGE on discontinuous 7.5% polyacrylamide slab gel.

Recording of T-Tubule Ca^{2+} Channels. Ca^{2+} channel activity from rabbit T-tubules was recorded from planar bilayers using the vesicle fusion protocol previously established for rat membranes (2). Vesicles were prepared essentially by the same procedure described above for antigen preparation (membrane fraction sedimenting at 22–27% sucrose after dissociation of triads in high salt concentration). Vesicles were stored at −80°C in 0.1 M KCl/0.3 M sucrose/5 mM Na-Pipes, pH 6.8. Planar bilayers were formed from 20 mg of lipid per ml of decane solution. An equimolar mixture of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Biochemicals) was used in all experiments. Membrane capacitance was 150–300 pF (300-μm-diameter bilayer cup aperture). Current records were low-pass filtered at 50–100 Hz using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). All recordings were made using a List EPC-7 patch clamp electrometer (List Electronics, Darmstadt, F.R.G.) at a holding potential of 0 mV in cis (voltage command side) 100 mM $BaCl_2$/50 mM NaCl/0.1 μM Bay K8644 and trans (ground side) 50 mM NaCl. Solutions were buffered with 10 mM Hepes-Tris, pH 7.2/0.1 mM EDTA. PN200-110, a DHP antagonist, was added to the cis side from a 10 mM stock solution in methanol. Channels usually insert with the cytoplasmic end in the cis chamber and the external end in the trans chamber (26). Thus, Ba^{2+} current at 0 mV was recorded as flowing outward through the channel. Antibodies were screened by comparing records of 120 s in control conditions and comparable or longer sampling times after the addition of antibody to the cis or trans bilayer chamber. Records were digitized at 2 points per ms, stored in 10 megabyte Bernoulli magnetic disks (Vufax, Decatur, GA) and analyzed on an IBM/AT computer as described (27, 28).

RESULTS

Localization of mAb21-4 in T-Tubules. The rabbit skeletal muscle membrane fraction used in this study expressed a high number of specific DHP binding sites (see *Materials and Methods*), suggesting that it was enriched in T-tubule membranes (13). mAbs against the T-tubule enriched membrane fraction were produced by immunizing BALB/c mice and subsequently fusing their spleen cells with P3 × 63Ag.8.6.5.3 mouse myeloma cells.

Using a solid-phase ELISA to screen for IgG production, we identified in one fusion 63 colonies/1056 total outgrowths that produced mouse IgG. Antibodies from these 63 hybridomas were examined for binding potential on two different membrane types. Vesicles from fractions enriched respectively in T-tubules and SR membranes were used as antigens on separate 96-well ELISA microplates. At this second level of screening, we found 15 colonies that produced antibodies against an antigen present only in the T-tubule-enriched fractions. The 63 IgG antibodies were also examined by immunocytochemistry with the goal of identifying a T-tubule epitope. Because the triads are located in rabbit skeletal muscle at the level of the A-I band junctions, we selected hybridomas producing antibodies that exhibited a striated granular doublet pattern at the light-dark band interface comprising the A-I band junctions in longitudinal sections (Fig. 1*a*). We selected against antibodies that stained the cell

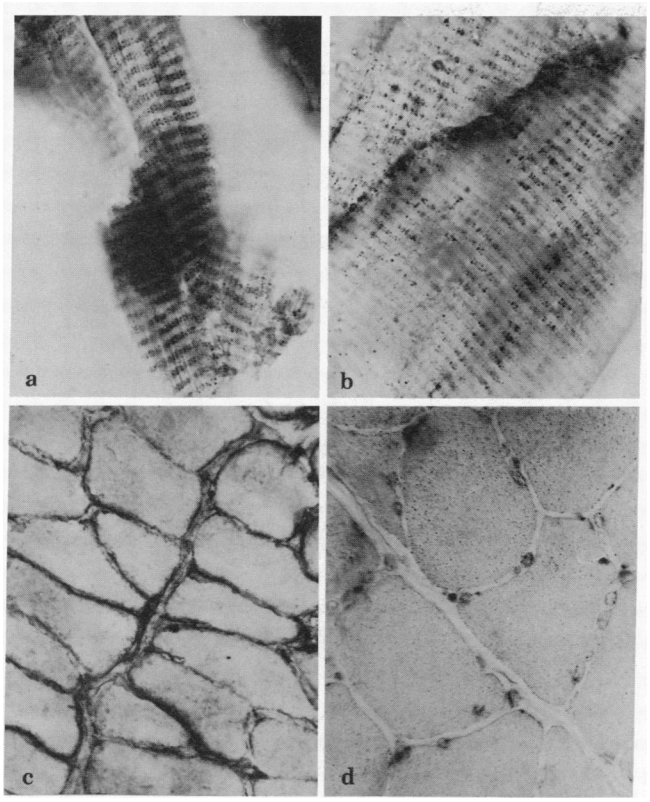

FIG. 1. Immunocytochemical staining pattern of mAb21-4. Muscle was clamped *in vivo* with a muscle clamp and fixed in 2% paraformaldehyde in P_i/NaCl, pH 7.4. Sections were stained via avidin–biotin-complex immunoperoxidase and biotinylated horse anti-mouse immunoglobulin using mAb21-4 as the primary antibody. A cross-striated doublet pattern is evident at the A-I junctions (light–dark band interface) consistent with the location of the triads in rabbit (*a*, *b*). mAb21-4 did not stain the plasmalemma on cross sections (*d*). The staining pattern of another antibody (Ab 40) is included to demonstrate plasmalemma staining pattern (*c*). (*b*, ×240; *a*, *c*, and *d* ×160.)

membrane (Fig. 1*c*). The findings of the immunoperoxidase assay and from the second ELISA were combined and used to narrow the penultimate selection to six hybridoma colonies for limiting dilution cloning. Our final criteria for selecting a hybridoma for cloning was the production of an antibody of the IgG isotype that exhibited the following: (*i*) high affinity for T-tubule-enriched membranes by ELISA; (*ii*) doublet granular peroxidase staining pattern at the level of the A-I junctions (Fig. 1 *a* and *b*); (*iii*) absence of staining at the plasmalemma of muscle cut in cross section (Fig. 1*d*); and (*iv*) changes in kinetics of Ca^{2+} channel activity (see below).

Two hybridomas fulfilled the above criteria (hybridomas 21 and 24); however, only one (hybridoma 21) was successfully cloned by limiting dilution. Clones that exhibited active growth were screened by all the above assays. In this communication the results from one clone (mAb21-4) out of six that satisfied our screening criteria are illustrated. mAb21-4 was of IgG_1 isotype with κ light chains and bound to T-tubule-enriched membrane fractions. Reaction with membranes from fractions enriched with SR was not detected. mAb21-4 exhibited a doublet staining pattern at the A-I junction by immunoperoxidase stain (Fig. 1 *a* and *b*). In contradistinction to antibodies produced by another hybridoma, (hybridoma 40, Fig. 1*c*) mAb 21-4 did not stain the plasmalemma on cross section (Fig. 1*d*).

Immunopurification. mAb21-4 was produced at ≈4–5 μg/ml in culture fluids. The antibody was purified on Protein A–Sepharose beads and used for immunoprecipitation at an approximate ratio of antibody/antigen (10:1) (wt/wt). M_r of the antigen seemed to be within the range of 170,000 as previously published (6, 7). The antigen was eluted in solubilizing buffer between 1 M and 2 M KCl (Fig. 2, lane B). We estimated the M_r of the eluted protein after disulfide bond reduction by $NaDodSO_4$/PAGE and silver staining. In a series of four experiments mAb21-4 consistently precipitated polypeptides of M_r values >175,000; 90,000; 55,000; and 34,000 (Fig. 2, lane B). Bands corresponding to major well-characterized muscle membrane proteins were also apparent on the gel from the eluted sample in Fig. 2. These proteins were present in large amounts in the T-tubule-enriched membrane fractions used as a source for the immunoprecipitation studies (ref. 18 and Fig. 2, lane A). However, we were able to eliminate these seemingly nonspecific contaminants in subsequent immunopurification experiments (data not shown) by first passing solubilized membranes on separate Affi-gel 10 columns on which albumin was immobilized.

Functional Effects of mAb21-4 on T-Tubule Ca^{2+} Channels. Activation of T-tubule Ca^{2+} channels by mAb21-4 followed by block of activity by the DHP antagonist PN200-110 is shown in Fig. 3. Kinetic effects of antibody were found more prominent under conditions of low basal activity elicited by submicromolar levels of Bay K8644. Under these conditions and after a short incubation period, mAb21-4 increases the frequency of openings as well as the mean open time. Hence, this effect is similar to the increase in channel activity observed with higher concentrations of agonist (2). There are numerous openings that in the presence of antibody appear to have a unitary conductance smaller than the average mean value; most of these appear to arise from fast opening transitions induced by the antibody that are filtered by the recording electronics. As shown in Fig. 3 *Right*, channels activated by mAb21-4 are blocked by the antagonist PN200-110 at similar doses reported for frog skeletal muscle Ca^{2+} currents *in vivo* (11). Thus mAb21-4 does not appear to compromise the DHP-binding site on the channel. Further quantitation of the kinetic effects of antibody is shown in Fig. 4. Open channel current histograms (Fig. 4 *Left*) show that the mean amplitude of openings in control channels after addition of mAb21-4 and after inhibition by antagonist is approximately the same. This is demonstrated by the fact that peak amplitudes (*x*-axis) for the control, mAb21-4, and PN200-110 conditions are all coincident. The larger amplitude (*y*-axis) of the histogram in the presence of mAb21-4, as well as the secondary small peak due to two simultaneously

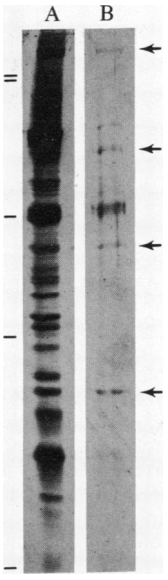

FIG. 2. Polyacrylamide gel electrophoresis. Acrylamide (7.5%) slab gels are 1.5 mm thick and they are silver stained. Sample buffer contains 5% (vol/vol) mercaptoethanol. M_r standards (–) (Pierce) 165,000; 155,000; 68,000; 39,000; and 21,500 are shown on the left of lane A. (Lane A) T-tubule-enriched membrane fraction. (Lane B) Eluates from immunoaffinity column between 1 M and 2 M KCl. The mAb consistently precipitates polypeptides that resolve into bands of ≥175,000; 90,000; 55,000; and 34,000, respectively (arrows). Unmarked bands appeared to be nonspecifically bound contaminants from major proteins present in original membrane fractions. We were able to eliminate these two bands in subsequent immunopurification experiments by passing solubilized membranes first on albumin immobilized on Affi-Gel 10 columns.

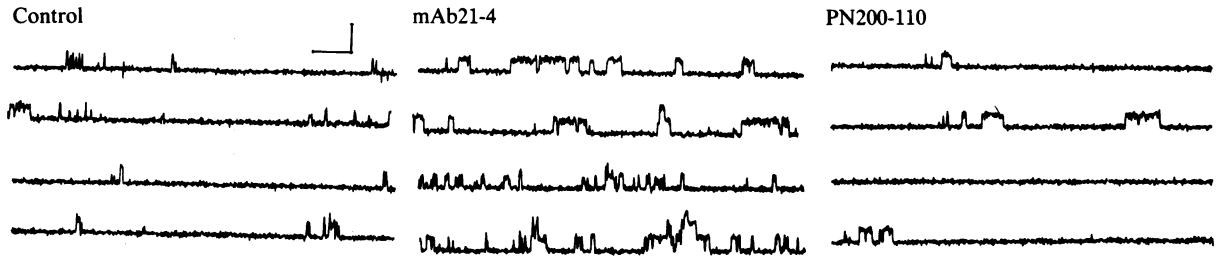

FIG. 3. Activation of T-tubule Ca^{2+} channels by mAb21-4. Blocks of four records (noncontiguous) are shown for the indicated condition. *Control* corresponds to activity elicited by 0.1 μM Bay K8644; *mAb21-4* is the continuation of the same experiment above after cis addition (and 120-s incubation time) of 1.8 μg (0.6 μg/ml, final concentration) of mAb21-4; and *PN200-110* is the continuation of the same experiment after cis addition (and 45-s incubation time) of 1 μM PN200-110. Calibration bars = 1 pA (vertical) and 400 ms (horizontal) are the same for all records.

open channels, reflects the increase in overall probability of opening elicited by the antibody. When open probabilities are scored as a function of time after incubation of channels with mAb21-4 (Fig. 4 *Right*) it is apparent that the antibody increases the frequency of both short and long events. During a period of 100 s at each condition, the records are broken into 100-ms segments, and the fraction of open time in each segment is shown as a vertical line of amplitude 0–1. Blank spaces are those in which no openings occurred. mAb21-4 increases the overall number of open channels, particularly those that give rise to lifetimes longer that the window size of 100 ms or longer—i.e., events with p = 1. This activity is clearly reversed by the antagonist PN200-110.

DISCUSSION

We report the production of a mouse mAb (mAb21-4) that modifies the kinetics of the voltage-dependent DHP-sensitive Ca^{2+} channel. The epitope recognized by the antibody is associated with polypeptides that resolve on $NaDodSO_4$/PAGE at M_r values of $\geq$ 175,000; 90,000; 55,000; and 34,000. This electrophoretic mobility is similar in part to that of polypeptides associated with the 1,4-DHP receptor. When the latter was purified with the aid of 1,4-DHPs, a large polypeptide of $\approx M_r$ 130,000–142,000 has been proposed to be linked to smaller components of 50,000 and of 32,000–33,000 with internal disulfide bonds (5–8).

Antibodies against the DHP receptors associated with the voltage-dependent Ca^{2+} channel have been previously prepared (29), but to our knowledge, none has modulated channel activity. The antibody reported here was prepared by immunizing mice with a native protein, prepared by T-tubule membrane fractionation. In preparations by others (29), the putative channel was isolated from $NaDodSO_4$-denatured proteins resolved on polyacrylamide gels. It is conceivable that under such conditions in the unfolding of protein following $NaDodSO_4$ treatment the molecule would lose conformationally active sites. Antibodies prepared against such denatured proteins may hence not affect function. Results from a previous fusion, for which we used native membrane proteins as antigens, yielded an antibody that also recognized an epitope specific to the T-tubule (16). In that work we

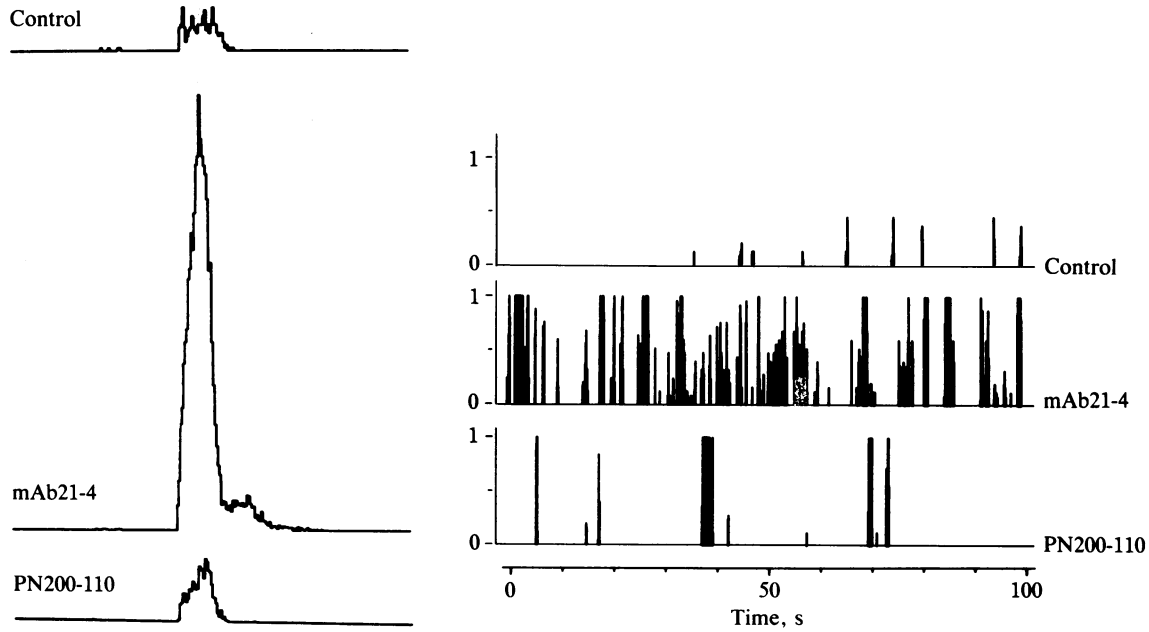

FIG. 4. Kinetic effects of mAb21-4 on T-tubule Ca^{2+} channels. Labels (Control, mAb21-4, and PN200-110) are for the same conditions described for Fig. 3. (*Left*) Amplitude histograms of open channels constructed by sorting 44,000 samples of current (22 s of continuous recording time) into 256 bins at a gain of 0.039 pA per bin. The *y* axis corresponds to bin content, and the *x* axis corresponds to the amplitude of open channel currents under conditions specified. Samples that fall into baseline current (closed channel current) were subtracted to allow proper scaling of those bins containing open channel current. Mean of the single peak current in control and PN200-110 and from the large peak in mAb21-4 was within 0.52–0.56 pA; the mean of the secondary peak in mAb21-4 (1.12 pA) is due to the occurrence of two open channels. (*Right*) Lifetime of single open channels monitored continuously during a period of 100 s. Multiple openings were excluded by appropriate setting of threshold detectors. Records were divided into consecutive segments of 100 ms. Lifetime of channels in each segment is given in units of 100 ms—i.e., lines of length 1 correspond to lifetimes > 100 ms. Blank spaces correspond to segments without open channels.

primarily screened with an immunocytochemical assay selecting antibodies that exhibited a peroxidase staining pattern in agreement with a T-tubule antigen localization. Used alone, the assay seemed to preferentially identify antibodies of the IgM isotype. Because of their size ($\approx M_r$, 1×10^6), such antibodies produced high nonspecific background noise in some studies. In this study we circumvented the problem by initially screening with a heterologous antibody that recognized the Fc region, characteristic of IgG types, of the antibodies produced by the hybridomas.

The physiological interactions between the Ca^{2+} channel and mAb21-4 were best evaluated within 20 min after the initial exposure time of the antibody to the antigen. This observation is consistent with the kinetics described generally for mAb binding to membrane antigens (30). The association between the latter and their corresponding mAbs has been described to be time dependent. This suggests to us that mAb21-4 bound specifically to an antigenic determinant and modified the channel function in the phospholipid bilayer system.

Surface membranes of an adult skeletal muscle cell consist of the plasmalemma that forms a sheath around the myofiber and a surface-connected intracytoplasmic network, the T-system or T-tubule (31). One significant difference between these surface membrane domains is the preferential localization of the Ca^{2+} channel in the T-tubule membrane (13, 14). Though the role of the Ca^{2+} channel in controlling secretion of hormones, release of neurotransmitters and Ca^{2+} availability in contraction of smooth and cardiac muscles has been recognized (32), its function at the level of the T-tubule in excitation–contraction coupling of skeletal muscle is not clear; mAb21-4 may help to elucidate this role.

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